

JOURNAL OF AVIAN BIOLOGY

Article

Egg colouration predicts brood size, telomere length and body condition of spotless starling fledglings

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Journal of Avian Biology

2018: e01686

doi: 10.1111/jav.01686

Subject Editor: Alexandre Roulin

Editor-in-Chief: Jan-Åke Nilsson

Accepted 29 March 2018

Understanding the impressive interspecific variation in avian eggshell colouration has attracted the attention of evolutionary ecologists for more than a century. Several functional explanations predict positive covariation between eggshell pigmentation and phenotypic quality of nestlings. We test this prediction in spotless starlings *Sturnus unicolor* by using biometric measurements and telomere length of hatchlings and fledglings as proxies of phenotypic quality. Female spotless starlings lay immaculate blue-green eggs, a sexually selected signal directed to males. Pigmentation predicts positive associations with concentration of antioxidants and testosterone in the yolk and with paternal provisioning effort during nestling growth. Eggshell pigmentation (blue-green chroma) is not associated with telomere length of hatchlings, which suggests weak maternal effects on this trait. However, we find negative associations of eggshell colouration with both body condition and telomere length of fledglings. Moreover, we find positive associations between eggshell colouration and clutch size, which suggests that sibling competition is higher in nests with more coloured eggshells. Previous works demonstrated that level of sibling competition is positively related to telomere erosion and, thus, the detected negative associations between eggshell colouration, body condition and telomere length of fledglings would reflect higher level of competition in nests with more coloured eggshells. We therefore speculate with the possibility that females that lay larger clutches also lay more coloured eggshells that elicit increased paternal provisioning effort and, thus, raise larger broods at the expense of telomere erosion of their offspring.

Keywords: brood size, clutch size, female ornamentation, sexual selection, sibling competition

Introduction

Understanding the impressive interspecific variation in avian eggshell colouration has attracted the attention of evolutionary ecologists for more than a century (Birkhead 2016). Several functional hypotheses have been proposed to explain its evolution



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with the consensus that a unique explanation is unlikely (Underwood and Sealy 2002, Kilner 2006, Hanley et al. 2013). Pioneering investigations on the role of eggshell colouration focussed on its obvious selective value in terms of mimicry or crypsis within its environment, leading to a decrease in predation risk (Cott 1940, Solis and de Lope 1995). Similarly, mimicry of brood parasitic eggs to those of their hosts reduced probability of parasitic egg detection and ejection by hosts (Baker 1923, Soler et al. 2003). Eggshell colour patterns might serve to identify own nests and eggs, which is crucial in the context of colonial bird species (Birkhead 1978), or for hosts of brood parasites in parasitized nests (Øien et al. 1995, Soler and Møller 1996). More recently, eggshell pigmentation has also been proposed to directly or indirectly affect offspring viability.

Two different groups of hypotheses suggest that egg colouration and offspring survival prospects are related in nature (Fig. 1). The first one is based on physical and antimicrobial properties of the two main pigments of eggshells, biliverdins and protoporphyrins, which would protect embryos from UV-light (Maurer et al. 2011) and/or pathogenic microorganisms (Ishikawa et al. 2010). The second one claims that eggshell colouration is a secondary sexual trait of females reflecting their phenotypic and genetic quality (Moreno and Osorno 2003). This hypothesis predicts positive associations between eggshell colouration and egg contents that would enhance offspring development. Support for this prediction has been found regarding antimicrobials (Morales et al. 2006), antioxidants (Navarro et al. 2011, Butler and McGraw 2013, Hargitai et al. 2016a, b) and steroid hormones (i.e.

androgens, López-Rull et al. 2010). However, antioxidants and androgens may have opposite effects on offspring viability since the former provide protection against oxidative damage and the latter increase oxidative stress (Royle et al. 2001, Alonso-Alvarez et al. 2007). Despite androgens effects might be detrimental early in life in terms of oxidative stress for developing embryos; these hormones enhance competitive behaviour of nestlings, which might ultimately overcome the early life effects (Gil 2008). Thus, the predicted positive effects of androgens in the eggs on offspring viability should mainly be apparent for fledglings, but not necessarily for hatchlings (Fig. 1).

The sexual selection hypothesis of eggshell colouration (SSHEC) predicts that males could invest differentially in reproduction depending on eggshell colouration (Moreno and Osorno 2003); a prediction that has received mixed support (Soler et al. 2008, English and Montgomerie 2011, Poláček et al. 2016). Thus, by its effects on parental investment, eggshell colouration would also positively influence nestlings' development (Fig. 1). However, the SSHEC also predicts that clutches of more coloured eggs could also be of larger size (Sanz and Garcia-Navas 2009, López de Hierro and De Neve 2010, but see also Hargitai et al. 2008, Węrzyn et al. 2011), which may have negative impacts on developing nestlings. This is mainly because mothers of high phenotypic quality are expected to lay eggs that are more blue-green pigmented (Soler et al. 2005, 2008, Cherry and Gosler 2010), but also larger clutches (Stearns 1992), which would increase sibling competition and, thus, negatively affect nestlings' growth (Sanz 1997). Thus, in the absence of

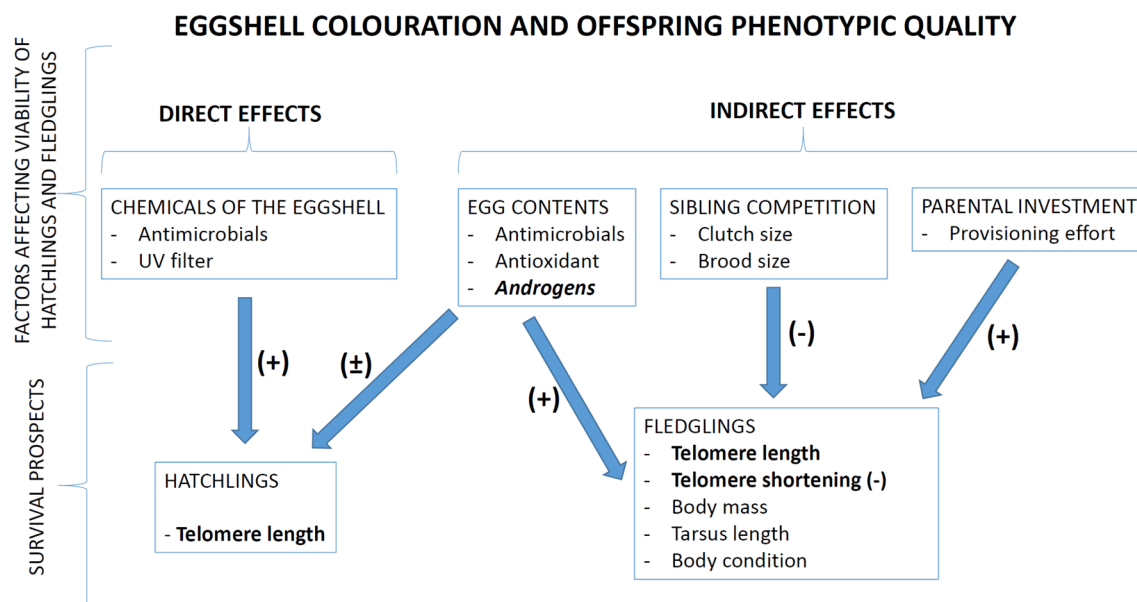


Figure 1. Diagram showing conceptual framework predicting associations between eggshell colouration and variables reflecting phenotypic quality of offspring. Direct mechanisms refer to chemicals of the eggshells, while indirect effects refer to consequences of eggshell colouration being a sexually selected character of females reflecting phenotypic and/or genetic quality. Indirect effects therefore include egg contents characteristics, level of sibling competition, and parental investment. Androgens will have negative and positive effects on embryo and nestling development, respectively, and it is highlighted in the diagram. Telomere length and dynamic are in bold font just to highlight that exploring their association with eggshell colouration is the most novel aspect of this study.

experimental manipulation, detecting the expected positive association between eggshell colouration and nestling growth or phenotypic quality in terms of survival prospects would largely depend on the strength of the association between clutch size and eggshell colouration. Here we explore the associations between eggshell colouration, clutch size, and phenotypic quality of hatchlings and fledglings in a wild population of spotless starlings *Sturnus unicolor* (Fig. 1).

Phenotypic quality is a complex concept that refers to phenotypic characteristics predicting survival prospects and/or reproductive performance of organisms (McNamara and Houston 1996). For nestling birds, immunocompetence and body size or condition have been traditionally used as proxies of phenotypic quality in terms of survival prospects because they are positively related to the probability of recruitment of fledglings into the breeding populations (Cichon and Dubiec 2005, Moreno et al. 2005a). Recently, telomere length and dynamics have also been proposed as proxies of survival prospects of free-living animals (Hausmann et al. 2005, Olsson et al. 2011, Barrett et al. 2013, Fairlie et al. 2016, Wilbourn et al. 2018). Telomeres are short repeats of the non-coding DNA sequence TTAGGG at the end of the chromosomes that protect integrity of genetic information during cell division (Blackburn 2001). When measured in nestlings close to fledging (hereafter fledglings), telomere length and dynamics mainly reflect number of cell divisions (Boonekamp et al. 2017), but also the stressful environmental conditions experienced by nestlings during development (Monaghan and Hausmann 2006, Monaghan 2014) as well as their probability of survival (Boonekamp et al. 2014, Watson et al. 2015, Salmón et al. 2016). Recent studies have reported negative associations between telomere length and early-life parasitic infection (Eisenberg et al. 2017), baseline corticosterone levels of nestlings (Hausmann et al. 2012, Quirici et al. 2016), and oxidative stress (Badás et al. 2015) as well as malaria infection of adult birds (Asghar et al. 2015). Telomere length therefore appears as a good proxy of health status and thus, of phenotypic quality of nestlings. Here, to test the predicted association between eggshell colouration and phenotypic quality of offspring, we used telomere length of hatchlings, and tarsus length, body mass, body condition and telomere length of fledglings as proxies of offspring phenotypic quality (Fig. 1). Previously published works have found support to this prediction, and a relationship between nestling body size or immunity and eggshell colouration has been detected for most (Moreno et al. 2005b, Soler et al. 2008, Hanley and Doucet 2009, Fronstin et al. 2016), but not for all (Fargallo et al. 2014) tested species. Thus, the novelty of our work is mainly to explore the predicted association between eggshell colouration and telomere length of hatchlings and fledglings as proxies of phenotypic quality at different nesting stages.

Some of the associations described in Fig. 1 have been explored in spotless starlings, which may facilitate the elaboration of predictions on the association between eggshell colouration and telomere length of hatchlings and fledglings. The spotless starling lays blue eggs, with blue-green

colouration being positively related to biliverdin concentration in the eggshells (López-Rull et al. 2008). Moreover, biliverdin concentration and eggshell colouration reflects concentration of antioxidants (Navarro et al. 2011) and testosterone (López-Rull et al. 2008) in the eggs. Thus, because of the possible negative effects of androgens on developing embryos (see above), the association between eggshell colouration and telomere length could depend on the balance between the effects of antioxidants and androgens during embryo development (and thus on hatchlings). We also know that phenotypic condition of females determines blue-green eggshell colouration in this species (Soler et al. 2008) and, thus, it is likely that nests with more coloured eggshells also harboured larger clutches and broods. In addition, we also know that colouration of spotless starling eggshells positively influences provisioning rates of males and immunocompetence of fledglings (Soler et al. 2008). Thus, telomere length, body mass, body condition and tarsus length of nestlings should also be positively related to eggshell colouration. However, in the case where eggshell colouration appears positively related to clutch size and/or brood size, sibling competition should also be higher in nests with more coloured eggshells and, thus, this may obscure the predicted positive association with phenotypic quality of fledglings. Negative effects of sibling competition on growth (Sanz 1997) and telomere length of fledglings have been detected in several species (Nettle et al. 2015, 2016, 2017, Costanzo et al. 2017, Cram et al. 2017). Thus, negative associations between eggshell colouration and body mass, body condition, tarsus length and telomere length are possible.

Starling nests used here were also subject to experimental manipulation of nest materials (green plants and nest lining feathers) known to affect telomere length and dynamics of nestlings (Soler et al. 2017). Briefly, the addition of feathers resulted in higher rates of telomere attrition, while the addition of green plants resulted in fledglings with longer telomeres in one of the studied populations. Thus, to test the aforementioned predictions, we considered experimental treatments as additional independent factors in our statistical models.

Material and methods

Study area, species and fieldwork

The study was performed in the Hoya de Guadix, southeast of Spain, a high-altitude plateau 1000 m a.s.l. with a semi-arid climate, during the 2012 breeding season. The spotless starling (hereafter starling) populations under study breed in cork-made nest boxes (internal height \times width \times depth: 350 \times 180 \times 210 mm, bottom-to-hole height: 240 mm) attached to tree trunks or walls at 3–4 m above ground. The two studied populations (hereafter locations) are located in the old railway stations of La Calahorra (37°15'N, 3°01'W) and Huéneja (37°13'N, 2°56'W), 20 km apart. Occupation of nest boxes was higher in La Calahorra than in Huéneja,

although empty nest boxes were available in both locations. For further information on the locations see Soler et al. (2017). Only first breeding attempts were considered in this study ($n = 52$).

The hole-nesting starling mostly breeds in colonies and use a variety of nesting materials, including feathers and green plants (Peralta-Sánchez et al. 2012, Ruiz-Castellano et al. 2016, Veiga and Polo 2016). In the studied locations, starlings start to lay their typical 4–5 eggs per clutch at mid-April. Full incubation typically starts with the penultimate egg resulting in asynchronous hatching (Soler et al. 2008). At the beginning of April, nest boxes were checked every second-third day until eggs were detected. Hatching date (age 0), defined as the day when half or more of the brood is hatched (Tomás 2015), was established by daily visits to nest boxes close to the expected dates (i.e. considering that incubation period lasts for 7–12 d after clutch completion). Nestling period ranges from 18 to 25 d (Veiga and Polo 2016).

Three days after hatching all nestlings in the nest were individually marked by cutting some of their down feathers from the head, back or wings. Moreover, for estimating telomere length, we collected a drop of blood from each hatchling by brachial venipuncture with the aid of a needle. Because of difficulties and risks associated with blood sampling in recently hatched nestlings, we just punctured their brachial vein and collected a small drop of blood in a blotting paper. Blood samples were kept dry at 4°C until DNA extraction three months later. On day 14 of nestling age, we recorded body mass (accuracy = 0.1 g) and tarsus length (accuracy = 0.1 mm) of nestlings and collected a second blood sample for estimating telomere length in 75 µl heparinized capillary tubes after puncturing the brachial vein. Blood was later stored in an Eppendorf tube with absolute ethanol and maintained at 4°C until analyses.

Eggshell colour measurements

We measured eggshell colour on three areas of the surface of the egg along the long axis: at both ends, and at the interception with the short axis (Avilés et al. 2006). Reflectance

spectra were obtained at 10 nm intervals from 360–700 nm for all eggs using a spectrophotometer (Konica Minolta Sensing [Seoul, South Korea], CM-2600d). Eggshells were illuminated at 45° to the measuring surface by a xenon light source, and the reflected light captured at the same angle. The measurements were taken relative to standard white (CM-A145, Konica Minolta Sensing) and dark references (CM-A32, Konica Minolta Sensing), used for calibration before measurement of each clutch. From spectrophotometric data, we calculated brightness as the mean reflectance calculated on reflectance values at every 10 nm interval across the entire spectrum (360–700 nm).

Our hypotheses do not deal directly with eggshell colouration as a signal, but as the result of pigment concentration. Thus, we did not consider retinal sensibilities of potential receivers and relied on eggshell colour variables extracted from spectrophotometric data that have proved successful in revealing female quality in previous studies (Siefferman et al. 2006, Navarro et al. 2011). As a variable indicating intensity of blue-green chroma, we used the proportion of total reflectance within the blue-green region (R400–575/R360–700) of the spectrum (Siefferman et al. 2006, Soler et al. 2008, Navarro et al. 2011). Biliverdin shows the lowest absorbance (Falchuk et al. 2002) within the blue-green region (400–574 nm), where reflectance of starling eggshells reaches its maximum (see Fig. 1 in Soler et al. 2008). Furthermore, we know that total reflectance in this region is related to biliverdin concentration in the eggshell and to female condition at laying (Moreno et al. 2006, López-Rull et al. 2008). Following Yezerinac et al. (1992), we estimated repeatability of colour measurements, either for the same eggs, or for mean values of eggs from the same clutch, which resulted in moderate repeatabilities (58.5–66.2%, Table 1). Thus, we used mean values for each clutch in subsequent analyses.

Telomere length estimations

DNA was extracted from blood samples using a standard chloroform-isoamyl alcohol based protocol (Ferraguti et al.

Table 1. Repeatability estimates of eggshell colour, biometry and telomere length measures within spotless starling nests. For egg colour measurements, we also show repeatability within spotless starling eggs. Repeatability was estimated following Yezerinac et al. (1992).

	MS-effects	MS-error	df	F	p	Repeatability
Colour measures						
Brightness						
Egg	18.70	2.72	192, 488	6.86	< 0.0001	66.23
Nest	151.66	18.70	50, 192	6.86	< 0.0001	60.21
BG-chroma						
Egg	2.32	0.41	192, 488	5.69	< 0.0001	60.99
Nest	17.74	2.32	50, 192	7.64	< 0.0001	58.53
Biometry						
Tarsus length	3.66	1.40	51, 90	2.62	< 0.0001	44.67
Body mass	191.8	110.0	51, 90	1.74	0.011	27.09
Telomeres						
Hatchlings	0.086	0.031	51, 88	2.73	< 0.0001	60.22
Fledglings	0.32	0.12	51, 87	2.67	< 0.0001	45.43

2013, Soler et al. 2015). DNA concentration was adjusted to 20 ng μl^{-1} using distilled water and conserved frozen (-20°C) until further analyses. The DNA quality was verified using a NanoDrop instrument. Relative telomere length of red blood cells (hereafter telomere length) was estimated by q-PCR following Criscuolo et al. (2009). We used the single copy gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as control to normalize the quantity of telomere sequence to the amount of DNA in the q-PCR reaction. The final PCR volume was 20 μl containing 10 μl of LightCycler 480 SYBR Green I Master (Roche) and 1 μl of DNA at 20 ng μl^{-1} of DNA. The reactions for telomeres or GAPDH were done in different plates due to the differential PCR conditions. Telomere PCR conditions were 10 min at 95°C followed by 30 cycles of 1 min at 56°C and 1 min at 95°C . GAPDH PCR started with 10 min at 95°C followed by 40 cycles of 1 min at 60°C and 1 min at 95°C , both performed in a LightCycler 480 RT-PCR System (Roche). Each sample was run in duplicate and samples with a coefficient of variation higher than 5% were removed from the analyses. Each 96-well plate included serial dilutions of DNA (40 ng, 10 ng, 2.5 ng, 0.66 ng of DNA per well) from a reference pool (the internal control) run in triplicate, which were used to generate the standard curves, and a blank control with no DNA. Quantification cycle values (Ct) were transformed into normalized relative quantities (NRQs) following Hellemans et al. (2007) procedure, which controls for the amplifying efficiency of each qPCR. Amplification efficiency for telomere products ranged between 1.858 and 2.143 and for the GAPDH product between 1.893 and 2.007. The slope of the calibration curve ranged between -3.718 and -3.021 for the telomere product and between -3.608 and -3.163 for the GAPDH product. CV calculated for a reference sample run in all plates was 1.90% for telomere RT-PCR and 0.33% for the GAPDH RT-PCR. The melting curves of q-PCR products confirmed no evidence of primer dimer or non-specific amplification. Therefore, the q-PCR approach provides a measure of telomere length relative to a single copy gene. Telomere restriction fragment (TRF) assay, which measures mean telomere length from fragments produced by digesting DNA with restriction enzymes that do not cut within the telomere sequence, is considered the 'golden standard' measure of telomere length. In comparison with q-PCR method, TRF is a technically demanding method, but allows interspecific comparisons (reviewed by Nussey et al. 2014). The method employed here is adequate to compare patterns of variation within species (for a similar approach see Asghar et al. 2015).

Estimated values of telomere length by the techniques explained above largely depend on the method of blood conservation and DNA isolation (Tolios et al. 2015, Reichert et al. 2017). Because of difficulties and risks associated to bleeding recently hatched nestlings, protocols of blood sampling and blood conservation for hatchlings and fledglings differed (dry in blotting paper vs in absolute ethanol, see above). A recently published paper compared

telomere length measurements by qPCR of blood samples stored both in FTA (Flinders Technology Associates) cards and frozen and concluded that there was not significant correlation between both estimates (Reichert et al. 2017). Thus, although most individuals were sampled at the two different ages, estimates of telomere length of hatchlings and fledglings cannot be directly compared. Telomere length was estimated for nestlings from 52 nests that reached the fledging stage ($n = 140$). Coefficient of variation of telomere length of three nestlings and one fledgling exceeded 5% and, thus, these estimates were removed from the analyses. For 137 nestlings, we gathered information of telomere lengths in both hatching and fledging stages.

Statistical analyses

Variation within clutches was significantly smaller than between clutches in terms of eggshell colouration, body mass, tarsus length, and telomere length, resulting in low to moderate intra-brood repeatability of measures (Harper 1994, range: 27.1–66.2, Table 1). We were not able to match egg and nestling identities and, since frequency distribution of these variables approached to Gaussian shape, we used average values per nest in subsequent analyses. Body condition was estimated as the raw residuals of average body mass of nestlings within the same brood after correcting for the effect of average tarsus length.

According to Soler et al. (2017), telomere length of starlings varied among study locations and depended on laying date in a non-linear shape. Thus, we included these variables in our statistical models. Furthermore, starling nests used here were also subjected to experimental manipulations of nesting materials (Soler et al. 2017). These experiments were performed after hatching and affected telomere length of starling nestlings (Soler et al. 2017). Thus, experimental manipulations of nest material (feather and aromatic plants) were also considered in our full initial models explaining nestling traits.

The relationship between eggshell colouration as independent variable, and telomere length of hatchlings or fledglings, or biometric variables of fledglings (tarsus length, body mass and body condition) as dependent variables, were explored in general linear models (GLM) (one per each phenotypic quality variable). These models also included experimental treatments and location as categorical predictors, and hatching date and square hatching date as covariates to control for possible non-linear effects. We also run models that included brood size or clutch size as additional independent factors. The two colour variables were strongly negatively related ($R^2 = 0.92$, Beta (SE) = -0.91 (0.06), $F = 226.00$, $df = 1, 50$, $p < 0.0001$) and, thus, both variables are essentially explaining identical variance of eggshell colouration. Arbitrarily, we show results using blue-green chroma as indicative of eggshell colouration. Results from models that included brightness instead of blue-green chroma provide qualitatively (i.e. statistical significance) identical results

with estimates having the contrary sign (Supplementary material Appendix 1).

As variables reflecting sibling competition, we used clutch size and brood size. These two variables are positively related ($R^2=0.26$, Beta (SE) = -0.56 (0.13), $F=17.89$, $df=1,50$, $p < 0.0001$) and, thus, we explored the expected associations with eggshell colouration in separate models. Results from these models depended on the used colour variable and, to avoid the strong co-linearity between the two colour variables (see above), the expected associations between clutch size or brood size and eggshell brightness and chroma were explored in separate GLMs. The models also included location as categorical independent factor and hatching date and square hatching date as covariates to control for potential non-linear effects.

For final model selection we used a backward stepwise procedure; i.e. fixed factors with the largest p-values were removed one by one up to p-values = 0.1 (Quinn and Keough 2002). Since we are mainly interested in testing the associations between eggshell colouration and telomere length of hatchlings and fledglings, the interactions between categorical factors (i.e. experimental treatments and locations) were not included in the statistical models. Moreover, interactions between locations and eggshell colouration were tested in separate models that also included main effects, while main effects were estimated in models that did not include interactions. All statistical tests were performed with Statistica 13.0 software (Dell-Inc 2015)

Data deposition

Data used in this paper can be found in CSIC Institutional Repository, with the accession numbers <<http://hdl.handle.net/10261/164410>>.

Results

Colouration of starling eggshells predicted telomere length (Beta (SE) = -0.35 (0.12)) and body condition (Beta (SE) = -0.32 (0.13)) of fledglings, but not telomere length of hatchlings, tarsus length, or body mass of fledglings (Table 2). Fledglings from clutches with more chromatic eggshells were those with shorter telomeres, and in poorer body condition (Fig. 2). The slope of the relationship between eggshell colouration and telomere length and body condition of fledglings was similar in both locations since interactions between location and eggshell blue-green chroma were statistically non-significant (Table 2). Neither clutch size ($p > 0.16$) nor brood size ($p > 0.094$) explained a significant proportion of variance when included as additional independent factors in the statistical final models with telomere length or body condition of fledglings as dependent variables (Table 2).

Eggshell brightness, but not blue-green chroma, was related to brood size (Fig. 2, Beta (SE) = -0.30 (0.13)), while a tendency was detected for clutch size (Fig. 2, Beta (SE) = -0.24 (0.14)) (Table 3). That was the case even after controlling for the effects of hatching date and location (Table 3).

Discussion

Some of the functional hypotheses previously proposed explaining eggshell colouration predict that more pigmented eggshells should result in better environments for developing offspring (Fig. 1). However, since eggshell colouration also predicts characteristics such as testosterone level in the eggs and intensity of sibling competition (i.e. clutch or brood size), some other theoretical scenarios predict negative effects on phenotypic condition of hatchlings and fledglings

Table 2. Initial and final models explaining telomere length (of hatchlings and fledglings), tarsus length, body mass and body condition of spotless starling nestlings. Blue-green egg colouration was used as eggshell colour variable. Numbers in bold indicate effects with associated p-values smaller than 0.1. Interactions between eggshell colouration (i.e. blue-green chroma) and location were estimated in separate models that also include main effects, while main effects were estimated in models that did not include interactions. Thus, degrees of freedom associated to F-values of the interactions between blue-green chroma and location explaining telomere length and body condition of fledglings are 1,46 and 1,47, respectively.

	Telomere length of hatchlings		Telomere length of fledglings		Tarsus length		Body mass		Body condition	
Initial model	F _{1,45}	p	F _{1,45}	p	F _{1,45}	p	F _{1,45}	p	F _{1,45}	p
Hatching date	0.70	0.408	4.28	0.044	0.18	0.671	1.48	0.230	1.50	0.227
(Hatching date) ²	0.56	0.458	3.43	0.071	0.34	0.563	2.14	0.150	1.98	0.166
Blue-green chroma	1.26	0.267	7.10	0.011	0.07	0.792	1.85	0.181	4.36	0.043
Location	0.18	0.676	13.82	0.001	6.31	0.016	11.14	0.002	4.25	0.045
Feather treatment	1.86	0.179	0.02	0.886	0.07	0.796	0.24	0.622	0.85	0.362
Plant treatment	0.13	0.719	1.00	0.323	3.94	0.053	0.98	0.327	0.35	0.560
Final model			F _{1,47}	p	F _{1,48}	p	F _{1,49}	p	F _{1,48}	p
Hatching date	–	–	4.59	0.037	–	–	–	–	–	–
(Hatching date) ²	–	–	3.70	0.060	4.68	0.035	9.12	0.004	5.78	0.020
Blue-green chroma (1)	–	–	9.10	0.004	–	–	–	–	5.66	0.021
Location (2)	–	–	14.21	< 0.001	6.77	0.012	9.53	0.003	3.26	0.077
Feather treatment	–	–	–	–	–	–	–	–	–	–
Plant treatment	–	–	–	–	4.313	0.043	–	–	–	–
(1) × (2)			0.14	0.710					2.72	0.106

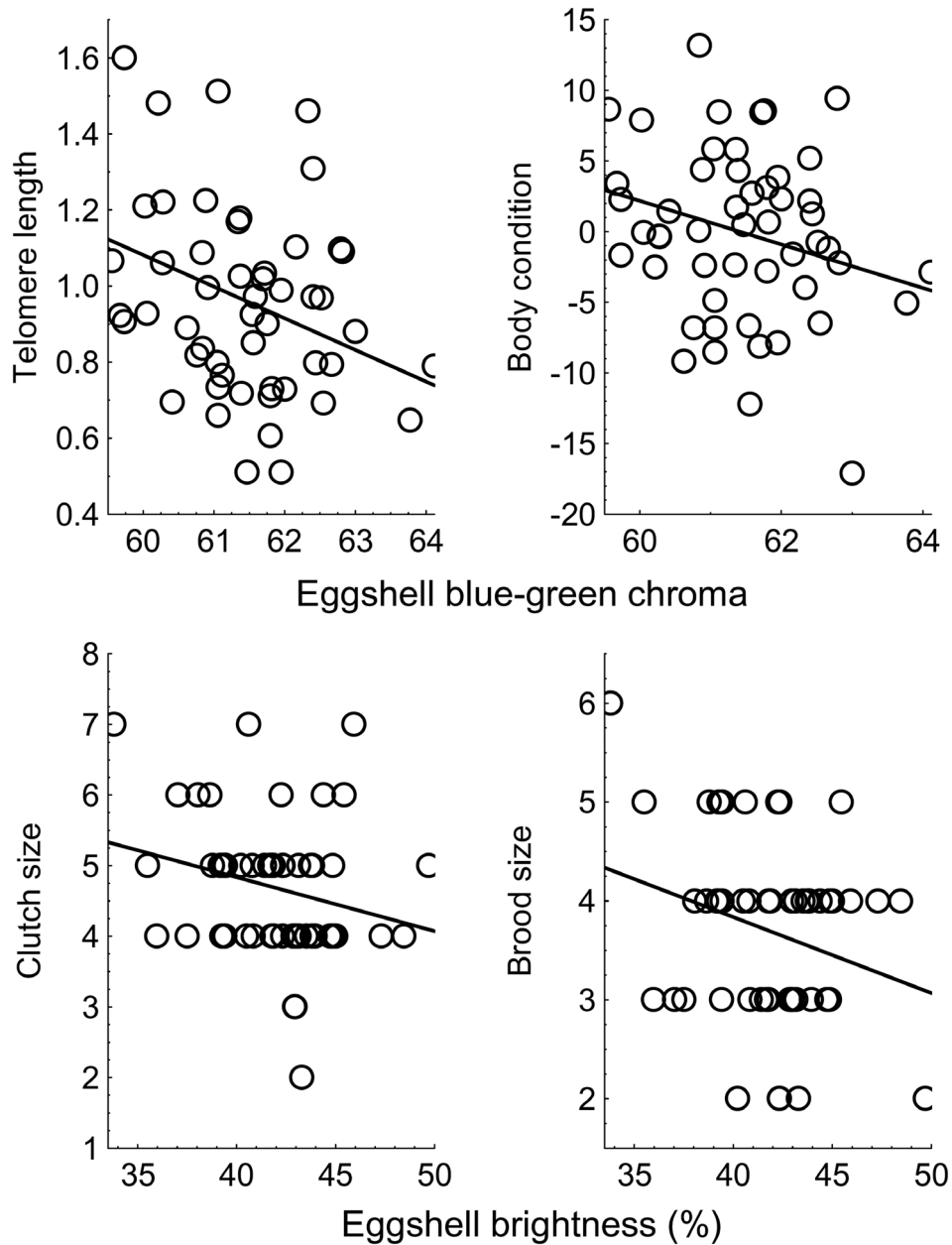


Figure 2. Relationship between within-nest mean values of blue-green chroma of spotless starling eggshells and telomere length and body condition of fledglings, and eggshell brightness and clutch size and brood size. Regression lines are shown.

respectively (Fig. 1). Thus, the strength and the sign of the association between eggshell colouration and phenotypic quality of nestlings will depend on the relative importance of factors related to eggshell colouration that affect measured traits of hatchlings and fledglings (Fig. 1). In this scenario, eggshell colouration of spotless starlings did not predict telomere length of hatchlings. Interestingly, smaller broods were associated with less coloured eggshells, suggesting a link between eggshell colouration and level of sibling competition. Moreover, we found negative associations between eggshell colouration and body condition and telomere length of fledglings, which suggest that these associations are mediated

by the relationship between eggshell colouration and clutch size, which is a proxy of the level of sibling competition experienced by spotless starling nestlings. Neither body mass nor tarsus length of fledglings was predicted by eggshell colouration. Below we discuss these results in different adaptive scenarios with particular interest on the role of androgens and sibling competition mediating the detected association between eggshell colouration and telomere length of hatchlings and fledglings.

Previous studies have found that eggs with more pigmented eggshells are those with higher concentration of antioxidants, vitamins, antimicrobials, steroid hormones and/or resources

Table 3. Results from general linear models testing the association between egg colouration (blue-green chroma in model 1 and brightness in model 2) and variables reflecting level of sibling competition. Numbers in bold indicate effects with associated p-values lower than 0.1.

	Clutch size				Brood size			
	Initial model		Final model		Initial model		Final model	
Model 1	$F_{1,47}$	p			$F_{1,47}$	p		
Hatching date	0.06	0.813	–	–	0.07	0.791	–	–
(Hatching date) ²	0.06	0.806	–	–	0.16	0.694	–	–
Blue-green chroma	0.30	0.584	–	–	1.95	0.169	–	–
Location	0.52	0.474	–	–	5.27	0.026	–	–
Model 2	$F_{1,47}$	p	$F_{1,50}$	p	$F_{1,47}$	p	$F_{1,48}$	p
Hatching date	0.22	0.642	–	–	0.19	0.662	–	–
(Hatching date) ²	0.25	0.623	–	–	0.35	0.558	3.72	0.060
Brightness	3.02	0.089	3.02	0.088	5.00	0.030	4.88	0.032
Location	0.69	0.409	–	–	5.85	0.020	5.75	0.020

(Morales et al. 2006, López-Rull et al. 2010, Navarro et al. 2011, Butler and McGraw 2013, Hargitai et al. 2016a, b). Most of these components buffer physiological stress and, thus, would positively affect fitness value of hatchlings and fledglings, being mirrored in longer telomeres. In the case of spotless starlings, we know from previous studies that yolk of more pigmented eggs contains antioxidants (Navarro et al. 2011) and testosterone (López-Rull et al. 2008) at higher concentrations than less pigmented eggs. Testosterone levels were positively associated with oxidative damage levels that resulted in higher rates of telomere erosion in European starlings *Sturnus vulgaris* (Stier et al. 2015). Consequently, antioxidants in the eggs might have counteracted the negative effects of testosterone during the embryonic phase, therefore explaining the lack of association between eggshell colouration and telomeres of hatchlings in our data set. Experimental manipulation of antioxidants and/or testosterone level in the eggs is in any case necessary to test this possible explanation.

Evidence of net beneficial effects of testosterone (Gil 2008, Noguera et al. 2011, Tobler et al. 2013) and antioxidants (Saino et al. 2003, McGraw et al. 2005) in the yolk in terms of competitive advantages and reduced oxidative damage for postnatal periods is extensive. Moreover, eggshell colouration of spotless starlings positively affects provisioning effort of males (Soler et al. 2008), which should benefit developing nestlings (Naef-Daenzer and Keller 1999). However, in spite of these related benefits, we failed to detect a positive association between eggshell colouration and the considered fitness-related traits of fledglings. Interestingly, eggshell colouration was positively associated with clutch and brood sizes, which suggests that sibling competition is higher in nests with more coloured eggs. The negative effects of sibling competition on developing nestlings is also well established (Sanz 1997), and it could explain the absence of positive associations between eggshell colouration and nestling phenotypes. For instance, we know that early life competitive disadvantages in terms of relatively low number of nutritional inputs and high begging effort during the nestling period accelerate telomere shortening and reduce telomere length of European starling nestlings (Nettle et al. 2015, 2017). Thus, telomere length and

brood size should be negatively related (Cram et al. 2017). In accordance with this prediction, an experimental increase of brood size caused a reduction in telomere length of barn swallows *Hirundo rustica* fledgling (Costanzo et al. 2017) and modulated the associations between telomere length and body size and immune development in European starling fledglings (Nettle et al. 2016). However, experimental modification of brood size in collared flycatchers *Ficedula albicollis* did not reduce telomere length of fledglings (Voillemot et al. 2012) and, thus, support to the predicted effect of sibling competition on telomere length is mixed. Our correlative results did not support the prediction either and we speculate with the possibility that the absence of association between brood size and telomere length may be due to females adjusting clutch size to the expected availability of resources for feeding the offspring during the nesting phase (Lack 1954, 1968). We did not manipulate brood size and, thus, nestlings in broods of different sizes may have experienced similar level of competition.

Eggshell colouration did not predict body mass or tarsus length of starling fledglings, whereas their body condition and telomere length were negatively related to eggshell colouration. Brood size did not predict telomere length or body condition of nestlings and, thus, the reduced brood size of nests with more brilliant eggs cannot explain the detected associations between eggshell colouration and body condition and telomere length of fledglings. Rather, the detected negative associations might be related to the effects of eggshell colouration enhancing provisioning effort of males (Soler et al. 2008). This is because reproductive success of females with large clutches would largely depend on the provisioning effort of their mates, and differential investment in eggshell pigmentation for such females would be of selective advantage. Based on the differential parental investment of males, we speculate that larger clutches with more coloured eggs would produce larger broods where more nestlings would compete for food at the expense of telomere and body condition deterioration. This possibility should be tested by means of experimental manipulation of brood size and eggshell colouration that males perceive during the egg stage.

Recently published articles support a link between telomere length and signalling characters in scenarios of sexual selection and parent–offspring communication. For instance, sexual differences in telomere dynamics have been detected in snakes (Rollings et al. 2017). In birds, associations between plumage colouration of males and telomere length and dynamics have been described (Parolini et al. 2017, Taff and Freeman-Gallant 2017). Moreover, in bluethroat *Luscinia svecica*, telomere length of social mates covaried positively suggesting assortative mating (Johnsen et al. 2017). Finally, signals of quality of barn swallow nestlings that are relevant to parent–offspring communication reflected their telomere length (Costanzo et al. 2017). All these examples deal with associations between signals of phenotypic quality of adult and nestling individuals and the telomere length of signalers. Our results are the first showing the expected association between a sexually selected signal of females and telomere length of their offspring, which is likely mediated by the association between the secondary sexual trait and fecundity. This has wide implications for understanding the evolution of signals, especially regarding those involved in parent–offspring communication (Morales and Velando 2013). Our results highlight the importance of incorporating telomere length estimations to test appropriately diverse adaptive hypotheses in scenarios of sexual selection and sibling competition.

Acknowledgements – We thank Olga Corona Forero, Francisco Miranda and Olaya García for technical assistance with DNA extraction from blood samples and amplification for telomere length estimation. We benefited from Manuel Martín-Vivaldi's comments on a previous version of the manuscript that greatly improved the paper.

Funding – Funding was provided by the Spanish Ministerio de Economía y Competitividad and European funds (FEDER) (CGL2013-48193-C3-1-P, CGL2013-48193-C3-2-P, CGL2012-30759, CGL2015-65055-P). JMP, GT and MRR were supported by Juan de la Cierva, Ramón & Cajal and Talentia postdoctoral contracts, respectively. JMP was partially supported by a 2017 Leonardo Grant for Researchers and Cultural Creators, BBVA Foundation. The Foundation accepts no responsibility for the opinions, statements and contents included in the project and/or the results thereof, which are entirely the responsibility of the authors.

Author contributions – JJS, GT, and JF designed the study with considerable assistance from JMP and CRC. CRC performed all molecular analyses with considerable assistance from JMP. GT and CRC performed most of the fieldwork with assistance by JJS and MRR. JJS performed all the statistical analyses and wrote the manuscript with substantial contribution from all authors.

Permits – The study was performed according to relevant Spanish national (Decreto 105/2011, 19 de Abril) and regional guidelines. The protocol was approved by the Ethics Committee of Spanish National Research Council (CSIC) and all necessary permits for nest and nestling manipulations were obtained from Consejería de Medio Ambiente de la Junta de Andalucía, Spain (ref: SGBY/FOA/AFR/CFS). Our study area is not protected but privately owned, and the owners allowed us to work in their properties. This study did not involve endangered or protected species. Time spent in each

starling nest was the minimum necessary and the protocols adhered to ASAB/ABS guidelines for the use of animals in research.

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Supplementary material (Appendix JAV-01686 at <www.avianbiology.org/appendix/jav-01686>). Appendix 1.